STAIN TECHNOLOGY

VOLUME 14 JANUARY, 1939 NUMBER 1

A TECHNIC FOR DIFFERENTIAL STAINING OF NUCLEOLI AND CHROMOSOMES[1]

C. S. SEMMENS and P. N. BHADURI, *Cytology Department, King's College, University of London, London, England*[2]

ABSTRACT.—**A procedure is described which enables a stain to be definitely located in the substance of the nucleolus. Material is fixed in either Navashin or Levitsky; the chromatin is stained by means of the improved Feulgen technic introduced by de Tomasi, and preparations brought thru the washing solutions down to distilled water. From distilled water the material is transferred to a mordant solution, 5% sodium carbonate in water, in which it is left for at least one hour. After mordanting wash well with water then stain for ten minutes in light green solution (90% alcohol, 100 cc, light green SFY, 0.5 g, aniline oil, 2 drops, well shaken); differentiate in alcoholic sodium carbonate solution, (70% alcohol saturated with carbonate); treat with 95% alcohol, absolute alcohol, equal parts xylene and absolute alcohol, clear in pure dry xylene and mount in neutral balsam. Cytoplasm and karyolymph should be quite clear, with magenta chromatin and well defined green nucleoli. The light green does not behave like a simple counterstain as in previous technics but as a definite stain for nucleolar material.**

Clear differentiation between chromosome material and the substance of the nucleolus is, at the moment, a matter both of interest and importance in connection with the various theories of the nucleolar functions now being widely discussed. The two types of substance usually associated with nucleoli and distinguished as chromatin and plastin, behave quite differently from the substance of the chromosomes when treated with certain chemical reagents. The simple method of differential staining to be described below was arrived at in the first instance from theoretical considerations of the

[1]The manuscript of this article was received for publication on March 10, 1938.

[2]Grateful acknowledgment is due to Prof. R. Ruggles Gates F. R. S. for the facilities to carry out this work in his laboratory at King's College, University of London.

probable chemical differences between the chromatin of the chromosomes and the substance of the nucleolus. The theory upon which the method is based suggests that altho the nucleoli of different plants and animals may vary somewhat in their response to the treatment, all should be stainable by slight significant variations of the technic.

Certain kinds of nucleoli are said to give a positive reaction with Feulgen's staining technic and also to stain quite readily with the ordinary mordanted nuclear dyes in common use. Ludford (1928) found that both oxyphil and basophil nucleoli in the oöcytes of mollusca gave negative results with the Feulgen test; he makes use of an ordinary counterstain and indicates that the chromosomes may contain other substances in addition to chromatin. The effects of various fixatives on the subsequent behavior of nucleoli towards stains have been observed by quite a number of workers, Sato (1928), Zirkle (1931), Shinke and Shigenaga (1933), Carlson (1936). Van Camp (1924) obtained some degree of differential staining by using Heidenhain's hematoxylin with Congo red and also the rather capricious Ehrlich-Biondi; Marshak (1931) used hematoxylin. Lenoir (1930), using basic fuchsin and malachite green, succeeded in staining the nucleolus; this stain, however, seems to have been fugitive.

There appears to be ample room for a more detailed study of the general behavior of the nucleolus towards fixatives, mordants and dyes. Preliminary considerations of some of the problems involved have led to the working out of the following simple technic for the differential staining of chromosomes and nucleoli. Attention is directed here to the distinction which is to be made between an ordinary general counterstain and a stain that can be definitely located in nucleolar material and cleared away from the rest of the cell. The results obtained up to the present are sufficiently attractive to warrant the publication of an outline of the practical procedure for the benefit of those likely to be interested in problems associated with the nucleolus.

Material may be fixed in either Navashin's, or Levitsky's solutions, preferably cutting down the acetic acid content to a minimum; the chromatin is then stained by means of the Feulgen reaction, using the improved technic introduced by de Tomasi (1936) and incorporating dye prepared according to the method given by Scanlan and Melin (1937), conversion of pararosanilin base (Gurr), C.I. 676, to the acetate, as Sample No. 13. For the subsequent special treatment the following solutions are required:

Sodium carbonate solution, 5% aqueous, (filtered).
70% alcohol saturated with sodium carbonate (filtered).
Solution of light green made up as follows: 90% alcohol, 100 cc.;

light green SF yellowish (Gurr), C.I. 670, 0.5 g.; aniline oil, two drops; shake well and use fairly fresh.

After staining by the Feulgen technic the material is given the following treatment from distilled water:

(1) Mordant for 1 hour in the aq. sodium carbonate solution.
(2) Wash in dist. water (leaving in this for about 30 min.).
(3) Stain for 10 min. or longer in the light green solution.
(4) Rinse with alc. sodium carbonate solution.
(5) Transfer to 95% alcohol and leave for at least 10 min.
(6) Examine under the microscope, if the cytoplasm is not free from green, rinse again in the alc. carbonate and transfer to 95% alcohol. The art of judging the correct differentiation can soon be acquired.
(7) Leave in abs. alcohol 10 min. or longer.
(8) Equal parts xylene and absolute alcohol, 10 min.
(9) Clear in pure xylene; (differential staining is more clearly defined after xylene).
(10) Mount in neutral balsam.

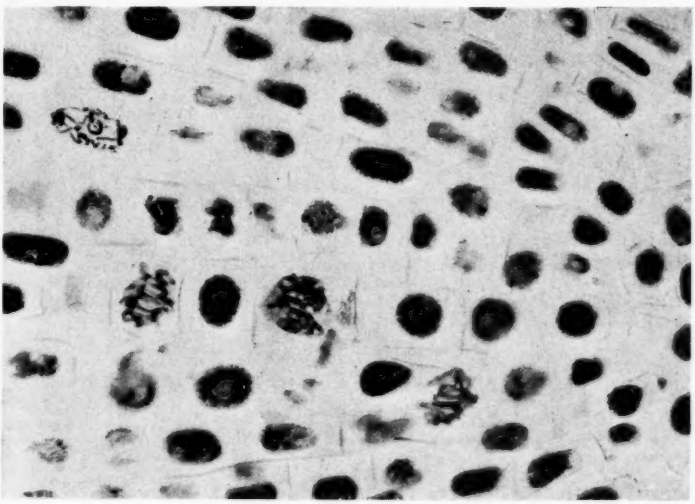


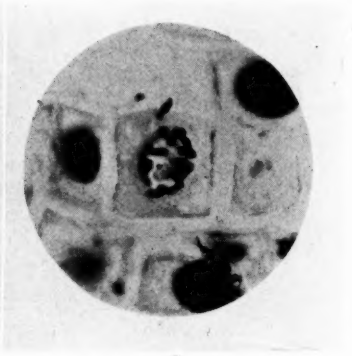
FIG. 1. Longitudinal section of the root tip of *Scilla nutans*, under low power to show nuclei in various stages. Chromatin has been stained magenta by means of the Feulgen reaction and nucleoli green by the new differential staining; cytoplasm and karyolymph are unstained.

Nucleoli are stained from light to dark green and are clearly demarked from the magenta chromosomes and colorless karyolymph. The characteristic color of the chromosomes, due to the Feulgen reaction, is neither masked nor debased by the green, which by careful

differentiation may be washed out from everything but the nucleolar material.

Normal eyes can distinguish very clearly between the colors red and green, but the difference, so easy to see, is extremely difficult to render in any marked degree of contrast with the available photographic materials. The accompanying figures show definite differences of tone between nucleoli and chromosomes, but they do not give an adequate impression of the brilliancy of the preparations or the sharpness of the color distinction. Fig. 1 shows a longitudinal section of Scilla root tip under low power and gives a general idea of

2



3

FIG. 2. Resting stages in root tip of *Scilla nutans* showing stained nucleoli with clear karyolymph. Chromatin threads have responded to the Feulgen reaction.

FIG. 3. Prophase in root tip of *Scilla nutans*. Chromatin deeply stained by the Feulgen. The differential staining of the nucleolus and satellite is clearly seen.

the effectiveness of the stain; Fig. 2 indicates how clearly the nucleoli are picked out in the resting stage and Fig. 3 shows a prophase in which may be plainly seen a chromosome with a satellite attached to the nucleolus.

The facilities which the technic offers for the critical study of relationships between nucleoli and chromosomes will be at once apparent, while there is every possibility that the method may also help in the elucidation of various interesting problems connected with fertilization.

REFERENCES

CARLSON, J. G. 1936. Effects of several fixatives on staining reactions in *Zea Mays;* especially with reference to the Feulgen reaction. Cytologia, **7**, 104–17.

DE TOMASI, J. A. 1936. Improving the technic of the Feulgen stain. Stain Techn., **11**, 137–44.

LENOIR, M. 1930. Coloration double en deux temps; Coloration double simultanée après fixation au picroformol alcoholique acétique, bichromaté. Compt. Rend. Soc. Biol., **104**, 1282–4.

LUDFORD, R. J. 1928. Studies in the microchemistry of the cell. Proc. Roy. Soc. B, **102**, 397–406.

MARSHAK, A. G. 1931. The morphology of the chromosomes of *Pisum sativum*. Cytologia, **2**, 318–39.

SATO, K. 1928. On the staining capacity of the nucleolus and its physiological significance. Folia. Anat. Jap., **6**, 717–36.

SCANLAN, J. T. and MELIN, C. G. 1937. The production of basic fuchsin suitable for the Feulgen technic. Stain Techn., **12**, 1–8.

SHINKE, N. and SHIGENAGA, M. 1933. A histochemical study of plant nuclei in rest and mitosis. Cytologia, **4**, 189–221.

VAN CAMP, G. M. 1924. Le rôle du nucléoli dans la caryocinese somatique. La Cellule, **34**, 7–50.

ZIRKLE, C. 1931. Nucleoli of the root tip and cambium of *Pinus Strobus*. Cytologia, **2**, 85–105.

CLEARING SPECIMENS FOR THE DEMONSTRATION OF BONE

R. W. Cumley, J. F. Crow, and A. B. Griffen, *Department of Zoology, University of Texas, Austin, Texas*

Abstract.—**A brief review is given of some of the older technics of clearing and staining various types of animals, in such a way that the bones are clearly visible in the surrounding tissues. The authors have modified some of these earlier practices. Particularly good results were obtained by combining the Schultze technic with that of Lundvall. Embryos are fixed in 95% alcohol, treated with 1% KOH, stained in 1:10,000 alizarin Red S, cleared in gylcerin, dehydrated with alcohol and further cleared in toluol, toluol saturated with naphthalene, and anise oil saturated with naphthalene. Details of the technic are presented in the text.**

Several technics have been suggested for rendering opaque objects transparent. In practically all cases the methods involve a depigmentation and dehydration of the tissue. One of the most successful applications of such technics is in the demonstration of skeletal parts, since the bones can be observed without dissection. Particularly in the field of embryology are such preparations useful, since they serve to present material for the study of ossification. The purpose of this paper is not so much to present new methods but rather to review some of the more successful older technics and to suggest some modifications which have been found to improve the quality of the results.

One of the best known methods is that of Spalteholz as reviewed by Sabin (see Guyer, 1930). In this method the embryo is bleached in hydrogen peroxide and cleared in benzol and synthetic oil of wintergreen. Some of the disadvantages of the technic are: (1) hydrogen peroxide causes the tissue to become filled with bubbles which are often difficult to remove, (2) hydrogen peroxide is not as efficient as a bleaching agent as are some other compounds, (3) the tissue tends to turn brown with age, and (4) oil of wintergreen causes the tissue to shrink. We have found that Batson's method of removing bubbles with a vacuum pump is desirable when the use of hydrogen peroxide cannot be avoided. Care must be taken, however, as the pressure within the tissues, created by evacuation of the surrounding area, often destroys or displaces the internal organs. Hydrogen peroxide is particularly useful in bleaching large insects whose chitinous cover-

ings are not disrupted by the bubbles (Stapp and Cumley, 1936). Sabin has modified the Spalteholz technic by fixing in Carnoy's fluid. This not only bleaches the tissue better than hydrogen peroxide, but also slightly swells the tissue and thereby counteracts the shrinking effect of the oil of wintergreen.

Another group of technics which are widely used are the methods of Schultze (1897) and Mall (1906) and modifications of these. In all of these procedures potassium hydroxide is used for bleaching and glycerin for clearing. Dawson has devised a method of staining bones with alizarin while clearing the soft tissues by Mall's method (Dawson, 1926). The disadvantage of using KOH is in its strong tendency to macerate tissue, particularly in embryonic material. Nevertheless, by carefully watching for maceration the danger can be avoided. The glycerin clearing method does not seem to produce the great transparency that is apparent in preparations cleared in the various oils. We have, however, obtained good results in using KOH and glycerin for clearing small fish, particularly if they are thin. Fish are not as easily macerated as embryos and hence are not likely to be injured by the strong alkaline solution. The Hollister (1934) technic for clearing fish is as follows:

1. Fix for several days in 70% alcohol.
2. Bath in dist. water for a few minutes.
3. Place in 1–4% KOH until the bones become apparent in the clear surrounding tissue.
4. Stain from 6–24 hr. or more, depending on the size of the specimen, in alizarin dye solution prepared as follows:

Alizarin, sat. solution in glacial acetic acid, 50%	5 cc.
Glycerin	10 cc.
Chloral hydrate crystals, 1% sol.	60 cc.

Place 1 cc. of the above solution in 1000 cc. of 1% KOH.

5. KOH, 1–20% without glycerin.
6. KOH, 1–4% with 40% glycerin.
7. Gradually increase the amounts of glycerin. The time between changes varies with the individual.
8. Store in glycerin.

Hollister recommends the use of ultra-violet light in the clearing process. This has the effect of bleaching and hastening the clearing. Two modifications of this technic may be suggested. First, we have found that by fixing in 95% alcohol instead of in 70% the chances of maceration are decreased. Second, alizarin red S dissolved in 1% KOH or in distilled water, in the proportion of 1 part of dye to 10,000 parts of the solvent, produces results quite as good as the more elaborate dye solution of Hollister. A photograph of a fish cleared by this modified Hollister method is shown.

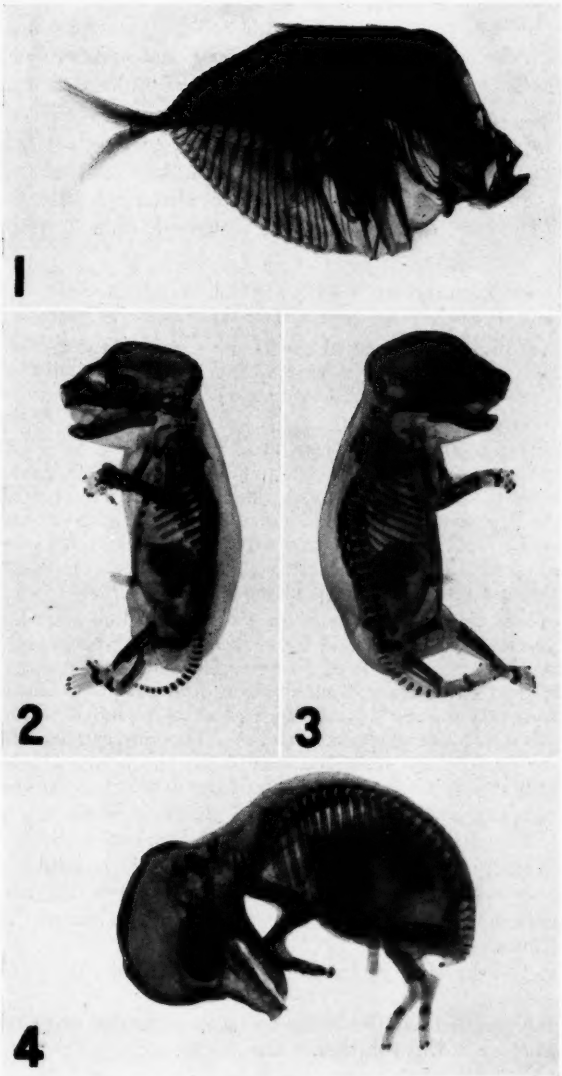


FIG. 1. FLATFISH

FIG. 2. SQUIRREL EMBRYO

FIG. 3. SQUIRREL EMBRYO

FIG. 4. PIG EMBRYO

All approximately natural size

For the demonstration of bony skeletons of mammalian embryos we have had the most success with a combination, with some modifications, of the KOH clearing methods and that of Lundvall (1927). Bleaching in KOH seems to produce better results than the oxalic acid which Lundvall recommends, and the danger of maceration is reduced by the addition of alcohol to the KOH when necessary. Toluol was substituted for benzene as a clearing agent because, while it clears equally well, it does not render the tissue so brittle (Galigher, 1934). The technic with which we have had the best results is as follows:

1. Fix for several days in 95% alcohol.
2. Wash in water a few hours.
3. Place in 1% KOH until the tissue surrounding the bones becomes clear and jelly-like. This requires from a few hours to a week or more, depending on the size and type of the embryo and the length of time fixed in 95% alcohol.
4. Stain in a solution of alizarin red S (alizarin sodium sulfonate) made up in the proportions of one part of dye to 10,000 parts of distilled water to which a few drops of dilute KOH have been added to make the solution basic. We have used the Coleman and Bell, Hartman-Leddon, and Eimer and Amend products. All were good, but none had Commission certification numbers on them. The embryo should remain in this solution for 3–12 hours.
5. Replace the dye solution with 1% KOH which contains about 10% glycerin. Gradually add equal amounts of glycerin and alcohol until the KOH has been completely replaced. During steps 3 to 5, if the embryo shows signs of maceration, gradually add alcohol until the concentration is 80% or 90%, and allow to harden a few days before resuming the clearing process. The embryo by this time should be completely destained except for the bone, which is red. Placing the embryo in sunlight hastens the decolorizing process.
6. Gradually add 95% alcohol until all the glycerin has been replaced. The changes must be gradual or shrinkage results.
7. Gradually replace the 95% alcohol with absolute alcohol. Change the solution several times to insure complete dehydration.
8. Replace the alcohol gradually with toluol. Change the toluol several times.
9. Change the embryo to toluol which has been saturated with naphthalene.
10. Replace this solution with anise oil saturated with naphthalene, and store in this solution.

Photographs of three embryos cleared by this method are shown. In these cases the viscera of the embryos were not removed, but in large embryos it may be necessary that this be done to obtain complete transparency. In large human embryos it has been suggested that the brain also be removed.

LITERATURE CITED

BATSON, C. V. 1921. The differential staining of bone. The staining of preserved specimens. Anat. Record, **22**, 159–64.

DAWSON, ALDEN B. 1926. A note on the staining of the skeleton of cleared specimens with alizarine red S. Stain Techn., **1**, 123–4.

GALIGHER, ALBERT E. 1934. Essentials of Practical Microtechnique. Albert E. Galigher, Inc., Berkeley, Cal.

GUYER, M. F. 1930. Animal Micrology. Rev. ed., pp. 103–4. University of Chicago Press, Chicago, Ill.

HOLLISTER, GLORIA. 1934. Clearing and dyeing fish for bone study. Zoologica, **12**, No. 10, 89–101.

LUNDVALL, HALVAR. 1927. Färbung des Skelettes in durchsichtigen Weichteilen. Anat. Anzeigerm **62**, 353–73.

MALL, F. P. 1906. On ossification centers in human embryos less than one hundred days old. Amer. J. Anat., **5**, 433–58.

SCHULTZE, O. 1897. Über Herstellung and Conservirung durchsichtigen Embryonen zum Studium der Skeletbildung. Anat. Anzeiger, **13**, Verhandlungen der Anatomischen Gesellschaft, pp. 3–5.

STAPP, PAUL and CUMLEY, RUSSELL W. 1936. A technic for clearing large insects. Stain Techn., **11**, 105–6.

THE USE OF THE FEULGEN TECHNIC WITH CERTAIN PLANT MATERIALS

THOMAS W. WHITAKER, *Division of Fruit and Vegetable Crops and Diseases, Bureau of Plant Industry, U. S. Department of Agriculture, Washington, D. C.*

ABSTRACT.—**The Feulgen technic as modified by Heitz promises to become an extremely useful tool in the solution of certain cytological problems. A procedure is outlined for using this technic with root tip smears, and smears of plant microspores. The chief improvement suggested over previous methods is that the material be mounted in euparal, after immersion in 95% alcohol. The technic is of value in the study of chromosome fragmentation, chromatid coiling, centromeres, etc., in both somatic tissue and in microspores.**

INTRODUCTION

The Feulgen technic, or so-called "nucleal reaction", has considerable promise as a cytological stain for both plant and animal materials. According to de Tomasi (1936) the stain was originally developed by Feulgen and Rossenbeck as a microchemical test. It depends upon the use of a reduced or colorless form of basic fuchsin which, upon contact with an aldehyde, develops a specific purple color.

Successful application of the Feulgen technic depends chiefly upon securing a suitable form of basic fuchsin. Conn (1937) states that the solution of this problem is at hand. Some intensive research into the chemistry and staining properties of this dye has indicated procedures that will give a satisfactory product (see Scanlan and Melin, 1937).

The writer has obtained basic fuchsin from commercial sources, manufactured specifically for use with the Feulgen reaction. Such products have proved to be entirely satisfactory and reliable.

The directions of de Tomasi (1936) should be followed very closely in making up the stain. It must be kept in mind that the reaction is contingent upon the ability of basic fuchsin to decolorize in the presence of sulfite, consequently the stain should not be used unless it has been completely decolorized.

The work of Margolena (1932) indicates that the Feulgen reaction can be used with a wide variety of botanical materials in cytological and histological studies. Heitz (1936) has devised a method of applying the Feulgen technic to root tip smears. He omits the sulfite

washes after staining, and substitutes 45% acetic acid. In acetic acid of this strength the stain does not remain in the cytoplasm. This eliminates the necessity for passing the material thru successive sulfite washes and a counterstain.

Klingstedt (1937) has had good success with grasshopper testes following the same general procedure as that used by Heitz. The principal difficulty has been to find a satisfactory method of making the slides permanent.

The writer has modified and extended the procedure used by Heitz, (1) by introducing methods of making the smears permanent; (2) by applying the technic to smear preparations of plant microspores.

With the Feulgen technic, the root tip smear method has been used with considerable success in obtaining chromosome counts where the

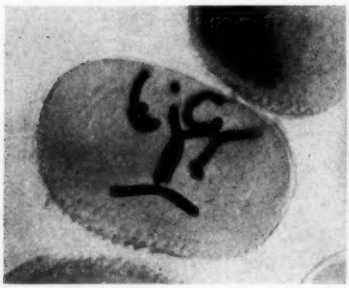


FIG. 1. Microspore of *Tradescantia paludosa* Anderson and Woodson. This photomicrograph shows the haploid number of 6 chromosomes + 4 fragments.

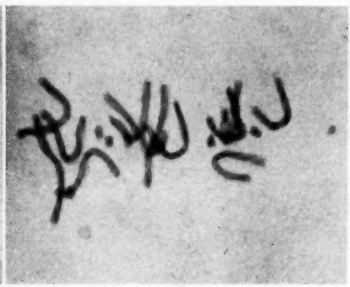


FIG. 2. Photomicrograph of a root tip cell of the same species, showing the diploid number of 12 chromosomes + 6 fragments. × 1200.

Thanks are due to Prof. Karl Sax, Biological Laboratories, Harvard University, for these photomicrographs.

material has small chromosomes as one finds in such genera as *Lactuca*, *Cichorium*, *Cucumis*, and certain species of *Asparagus*. It can also be employed with profit in studies of chromosome fragmentation, chromatid coiling, location of centromeres, secondary constrictions, and satellites where the material has large chromosomes (*Tradescantia*, *Lilium*, etc.; see Fig. 2).

As La Cour (1937) has stated, root tip smears are open to the criticism that it is often difficult to find a sufficient number of plates in polar view to make satisfactory counts of the chromosomes. Where the material has fairly small chromosomes, however, and where they are not too numerous as in most species of *Lactuca*, or where the chromosome number is small as in species of *Tradescantia*, root

tip smears making use of the Feulgen technic seem to have promise. Feulgen preparations of microspores of *Tradescantia*, *Aloe* and *Lilium* were found useful in the study of fragmentation, centromeres, etc., (see Fig. 1). The prophases of such material are extremely well fixed.

Procedure

Fixation: In the writer's experience it is quite important that a fixative be employed which contains a minimum of acetic acid. Benda's fluid (low acetic) made up according to the directions of La Cour is perhaps one of the best fixatives for use with the Feulgen reaction. Good results have been obtained by using Benda's fluid without acetic acid. Navashin's solutions can be used, but they do not give as uniformly good results as do fixatives containing osmic acid.

The time of fixation varies considerably with different types of material. For root tips, 15–20 minutes may be sufficient. For entire anthers with the enclosed microspores, 30–45 minutes may be required.

Hydrolysis: This operation takes place in *N* HCl at a temperature between 50–60° C. The length of time allowed for hydrolysis is one of the critical points of the technic. In general, it is governed by the length of the period of fixation, i.e., if fixation lasts 30 minutes, hydrolysis should extend over the same period.

Staining: After hydrolysis, the material is placed in the stain from 15–20 minutes, and then transferred directly to a dish containing 45% acetic acid (10–15 minutes or longer). The writer has found that some materials overstain easily, making it difficult or impossible to clear the stain out of the cytoplasm, and for this reason the material should not remain in the staining solution too long.

Mounting: After immersion in 45% acetic acid the material is transferred to a slide and placed in a drop of acetic acid of the same strength. Root tips are dissected into thin strips of tissue, if possible, only a few cells in thickness. Anthers are opened by an incision at one end, and the contents pressed out. The remnants of anther walls and other debris are removed. The cover slip is placed over the material, and pressure applied until it has reached the desired thickness. Considerable pressure can be applied to root tip smears without damage, but with microspores care should be taken so that individual cells are left intact. The slide should be heated to nearly the boiling point at least three times, some pressure being applied each time to the cover slip. This operation insures the material adhering either to the slide or the cover slip.

Making permanent: The cover slip is floated off in a mixture of equal parts of absolute alcohol and glacial acetic acid. Both cover slip and slide are transferred to 95% alcohol from this mixture. They are permitted to remain in this solution for at least 15 minutes and are mounted in euparal. The latter step must be carried out in a room where the relative humidity is low, and care should be taken to avoid breathing on the slide, otherwise there is danger of it becoming cloudy or white. The writer has slides prepared according to the directions outlined above which are over one year old, and show no signs of deterioration.

LITERATURE CITED

CONN, H. J. 1937. Recent advances in the standardization and improvement of biological stains. Bot. Rev., **3**, 72–83.

DE TOMASI, J. T. 1936. Improving the technic of the Feulgen stain. Stain Techn., **11**, 137–44.

HEITZ, E. 1936. Die Nucleal Quetschmethode. Ber. Deut. Bot. Gesellsch., **53**, 870–88.

KLINGSTEDT, H. 1937. Permanent Feulgen smears from previously fixed material. Stain Techn., **12**, 111–12.

LA COUR, L. 1937. Improvements in plant cytological technique. Bot. Rev., **3**, 241–58.

MARGOLENA, L. A. 1932. Feulgen's reaction and some of its applications for botanical material. Stain Techn., **7**, 9–16.

SCANLAN, J. T. and MELIN, C. G. 1937. The production of basic fuchsin suitable for the Feulgen technic. Stain Techn., **12**, 1–8.

MODIFICATIONS OF LEBOWICH'S SOAP-WAX TECHNIC

C. E. Moritz, *Department of Zoology, University of Idaho, Moscow, Idaho*

Abstract.—**Lebowich's technic is outlined for simultaneous dehydration and infiltration of tissues by a medium composed of stearic acid, 56° C. paraffin, diethylene glycol, and monoethanolamine. The prices and places where these materials may be purchased are given.**

Tissue for sectioning is placed in acetone, C.P., for 1 hour, then put directly into the soap-wax medium at 60° C. under reduced pressure, and finally embedded in new soap-wax.

Modifications include a simplification of the apparatus used by Lebowich. A preserving jar fitted with a rubber stopper serves as a vacuum chamber, and use of an aspirator accomplishes the reduction of pressure. With invertebrate embryos up to 1000 μ diameter no reduction of pressure is needed. Embryos are fixed, washed, placed in acetone, infiltrated in soap-wax, and embedded.

By this soap-wax method the alcohols, xylene, and overnight drying of affixed ribbons are eliminated. Tissue may be fixed, sectioned, stained, and permanently mounted within 6 to 8 hours.

In 1936 Lebowich[1] published a technic using soap-wax as a medium for simultaneous dehydration and infiltration of tissues. This technic with modifications has offered several advantages for sectioning molluscan embryos, and I believe others will find its application valuable.

Since the journal in which Lebowich published is one not usually read by biologists, it seems advisable to state Lebowich's general method while considering modifications of it.

The soap-wax medium is made from the following:

100 g. of XXX saponified stearic acid for research purposes, obtainable from Harkness and Cowing Co., Cincinnati, Ohio, at about $1 per pound.

400 g. of 56° C. paraffin, C.P.

7.5 g. of diethylene glycol, C.P., obtainable from City Chemical Corp., 132 West 22nd Street, New York City, at 50c. per 100 g.

7.5 g. of monoethanolamine, C.P., obtainable from Carbide and Carbon Chemicals Corp., South Charleston, West Virginia, at $1 per pound.

[1]Lebowich, R. J. 1936. Rapid, reliable and simple technic for the simultaneous dehydration and infiltration of human tissues. Arch. Path., 22, 782–805.

Making permanent: The cover slip is floated off in a mixture of equal parts of absolute alcohol and glacial acetic acid. Both cover slip and slide are transferred to 95% alcohol from this mixture. They are permitted to remain in this solution for at least 15 minutes and are mounted in euparal. The latter step must be carried out in a room where the relative humidity is low, and care should be taken to avoid breathing on the slide, otherwise there is danger of it becoming cloudy or white. The writer has slides prepared according to the directions outlined above which are over one year old, and show no signs of deterioration.

LITERATURE CITED

Conn, H. J. 1937. Recent advances in the standardization and improvement of biological stains. Bot. Rev., **3**, 72–83.

de Tomasi, J. T. 1936. Improving the technic of the Feulgen stain. Stain Techn., **11**, 137–44.

Heitz, E. 1936. Die Nucleal Quetschmethode. Ber. Deut. Bot. Gesellsch., **53**, 870–88.

Klingstedt, H. 1937. Permanent Feulgen smears from previously fixed material. Stain Techn., **12**, 111–12.

La Cour, L. 1937. Improvements in plant cytological technique. Bot. Rev., **3**, 241–58.

Margolena, L. A. 1932. Feulgen's reaction and some of its applications for botanical material. Stain Techn., **7**, 9–16.

Scanlan, J. T. and Melin, C. G. 1937. The production of basic fuchsin suitable for the Feulgen technic. Stain Techn., **12**, 1–8.

MODIFICATIONS OF LEBOWICH'S SOAP-WAX TECHNIC

C. E. MORITZ, *Department of Zoology, University of Idaho, Moscow, Idaho*

ABSTRACT.—**Lebowich's technic is outlined for simultaneous dehydration and infiltration of tissues by a medium composed of stearic acid, 56° C. paraffin, diethylene glycol, and monoethanolamine. The prices and places where these materials may be purchased are given.**

Tissue for sectioning is placed in acetone, C.P., for 1 hour, then put directly into the soap-wax medium at 60° C. under reduced pressure, and finally embedded in new soap-wax.

Modifications include a simplification of the apparatus used by Lebowich. A preserving jar fitted with a rubber stopper serves as a vacuum chamber, and use of an aspirator accomplishes the reduction of pressure. With invertebrate embryos up to 1000 μ diameter no reduction of pressure is needed. Embryos are fixed, washed, placed in acetone, infiltrated in soap-wax, and embedded.

By this soap-wax method the alcohols, xylene, and overnight drying of affixed ribbons are eliminated. Tissue may be fixed, sectioned, stained, and permanently mounted within 6 to 8 hours.

In 1936 Lebowich[1] published a technic using soap-wax as a medium for simultaneous dehydration and infiltration of tissues. This technic with modifications has offered several advantages for sectioning molluscan embryos, and I believe others will find its application valuable.

Since the journal in which Lebowich published is one not usually read by biologists, it seems advisable to state Lebowich's general method while considering modifications of it.

The soap-wax medium is made from the following:

100 g. of XXX saponified stearic acid for research purposes, obtainable from Harkness and Cowing Co., Cincinnati, Ohio, at about $1 per pound.

400 g. of 56° C. paraffin, C.P.

7.5 g. of diethylene glycol, C.P., obtainable from City Chemical Corp., 132 West 22nd Street, New York City, at 50c. per 100 g.

7.5 g. of monoethanolamine, C.P., obtainable from Carbide and Carbon Chemicals Corp., South Charleston, West Virginia, at $1 per pound.

[1]Lebowich, R. J. 1936. Rapid, reliable and simple technic for the simultaneous dehydration and infiltration of human tissues. Arch. Path., 22, 782–805.

The stearic acid and paraffin are each melted and then stirred together at 85° C. at high speed in a glass container, preferably by an electric mixer, for about 5 minutes. The amounts of diethylene glycol and monoethanolamine are added, and the mixture is stirred for an additional 30 minutes at high speed and at 85° C. Allow the heat to drive off the bubbles after stirring. When cooled, the medium resembles ivory soap in color and texture. Many of the chemical and physical properties of the soap-wax solution are published in Lebowich's paper.

The infiltrating apparatus used includes a short wide-mouthed jar fitted with a No. 13 rubber stopper. Thru the rubber stopper a glass tube is fitted tightly, and 3 or 4 feet of vacuum tubing is attached to the glass tube. During the process of embedding, the jar is placed in an oven to obtain the correct temperature. The vacuum tubing leads out of the oven to an aspirator attached to a faucet. By means of a glass Y-tube a vacuum gauge is attached along the line to denote the vacuum created by running the water thru the aspirator. Lebowich's apparatus is much more complicated and expensive, as he uses a motor driven vacuum pump and special vacuum chamber. The simpler and less expensive articles do just as well.

To embed tissue:

Fix in any ordinary fixative. If corrosive sublimate is an ingredient, the tissue must be washed in water or a low percentage of alcohol before placing the tissue in acetone.

Transfer to acetone, C. P. for 1 hour with a change of solution after 30 minutes. With molluscan embryos 600 to 1000 μ in diameter 30 to 40 minutes have been found to be sufficient with no change of fluid. The purpose of the acetone is to extract fats which are not penetrable by the soap-wax.

Place the tissue in a dish containing melted soap-wax. Put the dish into the wide-mouthed jar and plug the latter firmly with the rubber stopper. Set the vacuum chamber in an oven at 58° to 62° C.

Reduce the pressure to ½ atmosphere (400 mm. or 15 inches) with the aspirator. Take about 15 minutes for this reduction of pressure by twisting the faucet every 2 or 3 minutes until the desired reduction is reached.

Clamp the vacuum hose shut and allow the tissue to stand for 40 to 45 minutes. The melting point of the soap-wax is 49.3° C., but better infiltration is obtained if the temperature is kept at 56° to 62° C.

Unfasten the vacuum hose from the aspirator and by loosening the clamp allow a gradual return to normal atmospheric pressure in about 15 or 20 minutes.

Transfer the tissue to a new dish containing melted soap-wax.

Orient the tissue with a cat's whisker or pig bristle stuck into the end of a glass tube by sealing wax, and allow the mixture to harden.

There is no need for immersion in water as the soap-wax solution is non-crystalline.

Section in the usual way. The ribbon sections better at 5 than at 10 μ.

With small objects such as the invertebrate embryos mentioned, reduction of pressure in the vacuum chamber is not necessary.

Tissues stored in alcohol for a considerable length of time may go directly into the soap-wax solution from the alcohol, omitting the acetone.

If yolk offers undue difficulties, it will section satisfactorily if soaked 8 to 12 hours in a solution of 8 g. of phenol crystals in 100 cc. of alcohol of any percentage from 35 to 80. The block should be chilled before cutting.

To affix sections to slides when serial sections are not desired, Lebowich's method works well.

Float the sections on a bath of 2.5% aq. solution of gum arabic at 45° to 48° C.

After the sections have stretched sufficiently transfer them for about 10 seconds to a water bath to remove the excess soap-wax. This may be done by using a clean glass slide.

After 10 seconds dip the sections onto the proper position on a slide prepared as indicated below. A longer time on the water bath may cause the sections to break into bits. Wipe away the excess fluid.

Dry on a warming plate at 45° to 50° C. for 1 hour. The sections are then ready to stain.

For affixing serial sections the following procedure has been devised:

Place a few drops of unheated 2.5% solution of gum arabic on a slide prepared as indicated below and transfer the ribbons in proper order onto this fluid.

Place the slide on a warming plate at 45° to 50° C. and stretch the ribbons as is normally done with paraffin technic.

Drain the gum arabic solution from the slide by holding a cloth around one end of the slide and tilting the slide gently.

When most of the gum arabic solution has been absorbed by the cloth, drop 15 to 20 drops of dist. water heated to 45° C. at the upper end of the tilted slide. Allow the water to wash under the ribbons, carrying away the excess gum arabic solution. Drain off as much of the water as possible around the sections and then place the slide on a warming plate at 45° to 50° C. for 1 hour. The slide is then ready for staining. This procedure requires a little skill to prevent the sections from adhering to the cloth when the slide is tilted, but it is easily done after a trial or two.

Preparing slides with adhesive requires a gelatin solution, as the albumen method does not work well for soap-wax ribbons. The gelatin solution is prepared as follows:

Allow 0.25 g. of a good grade of granular gelatin (Lebowich recommends No. 850,669 granulated gelatin by Milligan and Higgins Corp.) to swell in 10 cc. of dist. water in an ice box for 30 minutes. Lebowich uses 0.5 g. The amount depends on the gram strength of the gelatin and must be determined by experiment.

Add 89 cc. of dist. water and 1 cc. of hydrogen peroxide.

Gently heat until the gelatin goes into solution.

Slides are prepared as follows:

Clean slides by immersion in a solution of equal parts of 95% alcohol and CCl_4. Allow the slides to evaporate to dryness.

Dip the cleaned slides into the gelatin solution.

Drain them so as to leave an even coating of gelatin. Dry.

Dip gelatin covered slides into a 5% solution of neutral formalin.

Dry and store for use.

The procedure for staining the sections on the slides is as follows:

Place slides with the ribbons affixed into CCl_4, C.P., for 10 minutes to dissolve the soap-wax.

Transfer to 95% alcohol, 3 to 5 minutes.

Two changes of tap water for a few minutes each.

Stain.

95% alcohol.

Two changes of 100%.

1 part xylene to 1 part 100% alcohol.

Mount in euparol.

The greatest difficulty with the technic is in the time and effort to obtain the materials needed. They are, however, of low cost. Once the soap-wax is made, the technic runs smoothly and has the following advantages over the ordinary paraffin methods:

Dehydration and infiltration are accomplished simultaneously.

The alcohols, or similarly used intermediate fluids, are omitted; and with minute objects the less changing of fluid the better.

Soap-wax will not crystallize when cooling.

Where difficulty in complete dehydration is encountered by alcohol or dioxan, such as is the case at marine stations, the soap-wax works remarkably well. Thus invertebrate embryos may be embedded directly instead of being left in fixing fluid where they often adhere to one another or collect debris before subsequent handling.

Soap-wax gives an excellent ribbon at 5 μ, in fact, it sections better at 5 than at 10 μ.

The technic is rapid and the work from living tissue to finished slide may be accomplished in 6 hours or less, if the materials needed are at hand. No overnight drying of slides is necessary.

STAINING PARAFFIN SECTIONS WITH PROTARGOL

3. THE OPTIMUM pH FOR REDUCTION. 4. A TWO-HOUR STAINING METHOD[1]

H. A. DAVENPORT, JANET MCARTHUR and S. R. BRUESCH,
With the Technical Assistance of Beulah F. Holland,

Department of Anatomy, Northwestern University, Chicago, Ill.

ABSTRACT.—**Further work on conditions affecting the reduction of paraffin sections impregnated with protargol showed that the optimum pH for sulfite-amidol mixtures was between 6.5 and 7.5. A staining method which requires about two hours to complete consists of the following steps: (1) One hour impregnation at 60° C. in 10% $AgNO_3$. (2) Wash in distilled water 3 changes of 30 sec. each. (3) Put into protargol (Winthrop Chem. Co., New York, N. Y.) 0.2% aq. for another hour at room temperature. (4) Rinse 2 sec. (5) Reduce one to two min. in amidol 0.2 g., Na_2SO_3 8 g., $NaHSO_3$ 1 g., and water 100 cc. (6) Wash thoroly. (7) Tone with 0.1% gold chloride. (8) Wash. (9) Reduce with a 0.5% aq. soln. of amidol (no sulfite). (10) Wash, dehydrate and cover. The method stains neurofibrillae and unmyelinated fibers and has worked well on most tissues of vertebrates. The stain follows acid alcoholic fixation.**

The use of protargol as a nerve fiber stain was discussed in a previous paper (Davenport and Kline, 1938). An improved fixative which consisted of trichloracetic acid, 10 g.; formic acid, 5 cc.; dissolved in a mixture of normal propyl alcohol and normal butyl alcohol (or of normal propyl alcohol alone) to make a final volume of 100 cc. was suggested as a modification of Hofker's fixative. The use of a second reduction after gold-toning, as a means of intensification of the stain, was discussed also. Subsequent experiments have led to the development of a staining method for axis cylinders which requires about two hours to complete. This gives, as a rule, better differentiated preparations than the previous procedure, which required about two days.

PART 3. THE OPTIMUM pH FOR REDUCTION

Since there are two reductions required in the staining process, the following comments apply to the first reduction, in which the protargol is reduced with an amidol-sulfite mixture.

[1]Contribution No. 280 from the Department of Anatomy, Northwestern University Medical School.

Paraffin sections of nervous tissue (dorsal roots, sciatic nerves and sympathetics of cats) were impregnated with 0.5% protargol for two days at room temperature, rinsed two or three seconds in distilled water and reduced with mixtures of 0.5% amidol in 5% sodium sulfite plus either acetic acid or sodium hydroxide to give a range in pH between 4 and 10. We had hoped that better differentiation of neural elements might be obtained by the acidification of the reducing solution. The advantages were slight, however, and were offset by the tendency for precipitate to form on the slide when the acidity of the reducer was near the isoelectric point of the protargol (about pH 4). The optimum for most material seemed to lie between pH 6.5 and 7.5 and was obtained by adding 0.5 cc. of glacial acetic acid to each 100 cc. of the amidol-sulfite mixture.

As mentioned in the preceding paper, differentiation was improved more effectively by reducing the concentration of amidol to 0.1% or 0.2% and increasing the sulfite concentration to 8 or 10%. This latter finding has been used in the rapid method to be described.

Part 4. A Two-hour Staining Method

The following method has some points in common with Foot's silver nitrate method (1932) in that 10% silver nitrate has been used for impregnation at a temperature above room temperature; a rather dilute solution of reducing agent used for the silver reduction; and a second reducing solution applied after toning. We have introduced a dilute protargol solution as a second impregnating medium, and found that its advantage lies in permitting the use of a buffered amidol-sulfite to secure good differentiation of nerve fibers, completeness of staining, and a minimum of trouble with precipitate.

The observation, described in the earlier paper, that nervous tissue fixed in Hofker's mixture or a modification thereof was readily impregnated by aqueous silver nitrate led to the present use of a combination of impregnations of silver nitrate and protargol in sequence. This procedure has improved the differentiation of nerve cells and fibers from connective tissue in all preparations and possesses the additional advantage of greatly shortening the time required for impregnation. An arbitrary procedure will be given, followed by comments on the various steps.

1. Fixation. Fix small nerves about two hours, large tissue blocks overnight, in the following mixture:

Pure formic acid (about 85% conc.)	5 cc.
Trichloracetic acid	5 g.
Normal propyl alcohol	25 cc.
Normal butyl alcohol	65 cc.

(To avoid esterification, mix just before using.)

2. Transfer to 95% ethyl alcohol then thru several grades of ethyl to water, allowing one to two hours for each change; dehydrate and embed in paraffin in the usual manner. Section and mount.

3. Remove the paraffin and descend thru graded alcohol to water.

4. Impregnate for about one hour in 10% aqueous silver nitrate (Merck's C.P. used) in a paraffin oven at 58 to 62° C.

5. Rinse thru three changes of distilled water, allowing the slides to remain about 30 sec. in each change.

6. Impregnate for an hour at room temperature in 0.2% protargol (Winthrop Chemical Company, New York City).

7. Rinse quickly (2 sec.) in distilled water and reduce in an amidol-sulfite solution made as follows:

Stock Solution A:	Sodium sulfite	10 g.
	Distilled water	90 cc.
Stock Solution B:	Sodium (or potassium) bisulfite	5 g.
	Amidol	1 g.
	Distilled water	95 cc.

For use mix 1 part of Sol. B with 5 parts of Sol. A. Reduction is complete in about one minute.

8. Wash in running tap water for several minutes, and transfer to one change of distilled water. (*Caution:* Do not put slides back to back as this will prevent the complete removal of the reducing agent.)

9. Tone for several minutes, until all the yellow color of the silver stain is replaced by gray, in a 0.1% aqueous solution of gold chloride.

10. Wash as in step 8.

11. Reduce with a 0.5 to 1.0% aqueous solution of amidol, preferably by dropping the solution on the slide. Sulfite must not be added. Reduction is complete in a few seconds.

12. Wash, dehydrate and cover.

Comments on Individual Steps of the Method

1. *Fixation.* Several variations in acid-alcohol fixatives have been tried and the experiments indicate that the stain will follow almost any combination of formic, acetic, or trichloracetic (or combinations of these) in either methyl, ethyl or propyl alcohol. The addition of formalin up to 2 or 3% in the solutions is optional, and, in a few instances, such an addition appeared to improve fixation and staining slightly. A mixture of 5% formic plus 5% trichloracetic acids in water, instead of the propyl-butyl alcohol mixture recommended, gave a good stain on a dorsal root. The trichloracetic acid concentration of 10%, previously recommended, appears to be stronger than necessary. After 50% pyridin fixation of spinal cord of cat,

many synaptic end-bulbs (*boutons termineaux*) were stained, but it appeared doubtful that the staining of these structures was complete. Aqueous 10% formalin, formalin-acetic, and Bouin's fluid have always given results inferior to fixatives based on a solution of trichloracetic acid.

2 and 3. These steps are ordinary routine and were not subjected to experiment.

4. The time, temperature, and concentration of the silver nitrate solution were found to be uncritical but interrelated. Impregnation for several days at 27° C. with a 2% solution gave results similar to those obtained at 60° with a 10% solution in 1 hour. Two hours in 10% silver nitrate at 60° followed by 20 hours at 27° did not cause overimpregnation.

Twenty hours at 60° C. caused excessive staining of connective tissue. From experiments with time intervals shorter than one hour, it appeared that 45 minutes (which included the warming of the solution from room temperature to 60° in the oven) was about the minimum. Solutions of silver nitrate stronger than 10% were not tried. Repeated use of the solutions was found to be practicable since those of 10% strength were used five or six times without apparent impairment of the staining reaction. Presumably they will continue to function properly as long as they remain clear.

5. Rinsing after treatment with silver nitrate was varied from a few seconds to three minutes. The best final impregnations seemed to occur when all excess silver nitrate was removed; hence a total time between one and two minutes has been recommended. The wash water must be distilled water and it should cover the slides completely. It is changed by pouring off and adding fresh water rather than transferring the slides from one container to the next.

6. We have attempted to secure staining by reducing immediately after impregnation with silver nitrate and subsequent rinsing, but this procedure was unsuccessful. Therefore, the second impregnation seems essential. Attempts were made to secure staining by using protargol alone at 60° for two hours, but these failed also, possibly because of too low concentration of the silver ion. After the original treatment with strong silver nitrate solution, however, neither the time of treatment with protargol solution nor its concentration is critical. We have tried varying the time of the protargol impregnation from a few minutes to an overnight period, and believe that at least 30 minutes at room temperature is necessary. Concentrations of 0.1, 0.2, and 0.5% protargol were used, but 0.2% seemed to be near optimum. Material left in the protargol more than one hour showed

no change of staining. We have preferred to use the protargol solutions only once altho they will function for several stainings.

7. Rinsing after the protargol impregnation was found to be optional. Neither of the reducing solutions keep indefinitely if exposed to air, but Sol. A is stable in stoppered bottles. We have not attempted to keep any amidol solutions for more than a few days, since they oxidize rather readily. The solution of amidol in bisulfite provides a buffered solution near neutrality when mixed with sulfite in proportions of 1:10 to 1:4. Differentiation of nervous elements was found to be better when dilutions of one part of Sol. B to 10 of Sol. A were used, but for some material the stain may be too pale. A solution of 0.2% amidol in 8 to 10% sodium sulfite may be used if desired with efficacy about equal to the sulfite-bisulfite mixture.

8. The reducing solution should be completely removed by thoro washing, otherwise rapid deterioration of the gold-toning solution occurs.

9. The gold-toning process is routine except that no hypo solution is used.

10. The washing at this stage merely removes the excess gold chloride solution. If omitted, the gold forms a fine precipitate on the slide during the second reduction, step 11.

11. Some preparations may stain with sufficient intensity to give what is wanted without reduction at this stage, but the stain is usually too pale, especially if 0.2% protargol is used for the final impregnation. Over-reduction does not occur if the excess gold chloride has been removed in the preceding step.

12. This step is routine.

Discussion

The foregoing method, with variations on the various steps, has been tried on spinal nerve roots, sympathetics, peripheral trunks, and a few specimens of the central nervous system of vertebrates. The following types were represented, but most of the material was from the cat: man, monkey, guinea pig, rat, cat, bull frog, and several species of fish. The general degree of success indicates that it has a rather wide range of application, altho, of course, it is not expected to cover all needs of a nerve stain. It has been less successful on large trunks of peripheral nerves than elsewhere, since the difficulty of obtaining perfect fixation seems to be the limiting factor rather than any inherent fault of the staining method.

This method has been checked with the two-day protargol method, previously described, by the direct comparison of slides made from

the same block of tissue and by fiber counts. Dorsal roots of cat, rat and monkey were cut serially and the numbers of fibers in the root found to agree, within experimental error, with the number of cells in the ganglion.

Cat, 4th cervical:	17,700	cells;	16,900	fibers
Rat, 13th thoracic:	5,500	"	5,600	"
Monkey, 1st sacral:	21,400	"	19,300	"

The unmyelinated fibers of the above nerve roots are extremely fine, and the test indicates that all, or nearly all, of the fibers were stained. Direct comparison of counts on three small fascicles of the cervical sympathetic nerves of a guinea pig, stained by both the short and long processes, gave the following:

	Number of fibers	
	2-day method;	*2-hour method*
Fascicle 1.	184	180
2.	644	668
3.	547	587

In this instance, also, the unmyelinated fibers were very fine and the agreement between the two methods gives a strong suggestion of complete staining.

In observations on other preparations stained by both methods it would seem that the 2-hour staining method is more likely to yield higher counts of nerve fibers than the 2-day method on account of greater discreteness of the individual fibers and greater clarity of the background.

REFERENCES

DAVENPORT, H. A. and C. L. KLINE. 1938. Staining paraffin sections with protargol: 1. Experiments with Bodian's method. 2. Use of *n*-propyl and *n*-butyl alcohol in Hofker's fixative. Stain Techn., **13**, 147–159.

FOOT, N. C. 1932. Two simple methods for the silver impregnation of nerve fibers in paraffin sections of the central and peripheral nervous systems. Amer. J. Path., **8**, 769–75.

THE PREPARATION OF PLASTER OF PARIS EMBEDDING BOXES

ARCHIE NORMAN SOLBERG, *University of Toledo, Toledo, Ohio*

There is a method of embedding with plaster of Paris boxes that does not seem to be commonly known, altho it has been used very successfully as a substitute for Guyer's[1] and Lee's[2] methods of embedding with paper boxes and Ferguson's[3] cellophane box. The technic is especially practical for large tissues and embryos, but serves equally well for small tissues. As the box is made of plaster of Paris, it is porous so that even within the box the paraffin is in contact with water, insuring even cooling, which results in a uniformly well-shaped

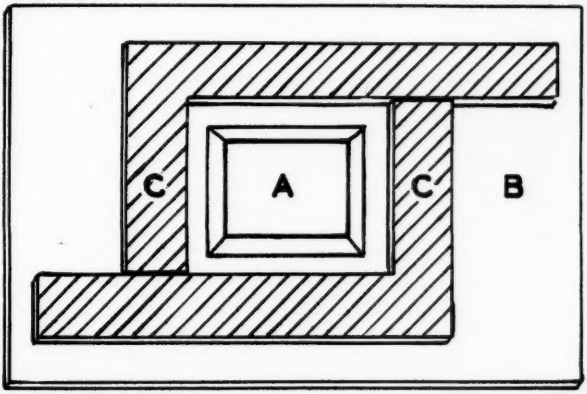


FIG. 1. Drawing of the mold used for preparing plaster of Paris embedding boxes. (A) Paraffin block, beveled, coated with a thin layer of vaseline, and sealed with the wide side down to (B), the copper plate. (C) Lead embedding angles. Any size box desired may be made by adjusting the embedding angles and size of the paraffin block.

block. Moreover, the method has the advantages of being very rapid and of saving the time one ordinarily uses in making paper boxes, for the box is permanent and needs only to be replaced when broken by rough handling. Boxes of various sizes can be made up at one's leisure (and will be ready to use whenever needed.)

The method of making them is as follows: Cut pieces of paraffin of

[1]Guyer, M. F. 1926. Animal Micrology, p. 37. Univ. of Chicago Press, Chicago, Ill.
[2]Lee, Bolles. 1928. Microtomist's Vade-mecum, pp. 74–75. P. Blakiston's Son & Co., Philadelphia, Pa.
[3]Ferguson, F. F. 1937. A cellophane embedding box. Stain Techn., 12, 71.

the size you desire the blocks. Bevel the paraffin block about 15° so that the bottom will be smaller than the top (Fig. 1, A) to allow the paraffin blocks to slip out when shrunk by cooling. Seal the block, wide side down, onto a copper plate (Fig. 1, B) or piece of cardboard and place around it two embedding angles (Fig. 1, C) about 1/8" from the widest part of the paraffin block. Coat the paraffin block with a thin layer of vaseline. Mix enough plaster of Paris and water to fill the mold and pour the mixture into the mold while still very wet. Allow the plaster of Paris to dry for an hour or more. Remove the embedding angles and when the plaster of Paris seems quite dry lift the box off the paraffin mold. When dry trim the box with a knife and sandpaper to the desired shape and thickness. It is advisable to leave the wall quite thick to prevent breakage.

To use the box: Soak it for a moment in the water to be used for cooling. Pick it out and pour out the water. Holding the box be-

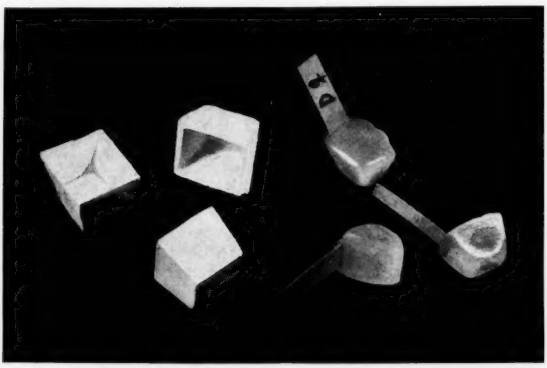


FIG. 2. Photograph of three plaster of Paris embedding boxes and three blocks made in them, showing how the blocks are labeled with a little paper tab.

tween the thumb and forefinger fill the box with melted paraffin using a medicine dropper. Drop the tissue into the melted paraffin. Orient tissue as desired with a warm needle or forceps. Insert a previously prepared slip of paper with identification marks on it into the melted paraffin at one end of the box (Fig. 2). Hold the box in the cold water to cool and blow gently on the surface until a thin film has congealed. Carefully lower the box under water and in a few seconds the paraffin will shrink from the sides of the box allowing the block to float free. The box may be used again immediately, but one can work faster by using two boxes alternately.

The use of plaster of Paris for making embedding boxes was first suggested to me by Margaret Long. Fred Schwind assisted in developing the technic for making them.

THE DEHYDRATION OF METHYLENE BLUE STAINED MATERIAL WITHOUT LOSS OF DYE

NORMAN D. LEVINE, *Laboratory of Animal Pathology and Hygiene, University of Illinois, Urbana, Illinois*

ABSTRACT.—**A method is given for dehydrating methylene blue stained protozoan smears which should be applicable to the dehydration of tissues stained *intra vitam* with methylene blue. The procedure is: Wash with distilled water, place in tertiary butyl alcohol for 1 to 2 minutes, then in three or more changes of tertiary butyl alcohol for 15 minutes to an hour each, and mount directly in balsam or pass thru two changes of xylene before mounting.**

The problem of dehydrating methylene blue stained tissues without excessive loss of dye has engaged biologists for a long time. No entirely satisfactory technic has been found.

In the course of some studies, in the Department of Zoology at the University of California, on the large flagellate protozoon, *Trichonympha collaris*, it was necessary to dehydrate methylene blue preparations with a minimum loss of stain. The best method found in the literature was that of Cole.[1] After staining, Cole fixed the tissues in 10% ammonium molybdate, and passed them thru water, absolute-alcohol-*n*-butyl-alcohol, *n*-butyl alcohol, and methyl-salicylate-xylene to balsam. Altho fairly satisfactory, this method entailed the loss of a great deal of dye.

In a search for a more suitable method, it was observed that dehydration could be effected in tertiary butyl alcohol with the loss of an insignificant amount of methylene blue. Fixed and stained smears were washed in repeated changes of distilled water for 15 minutes to an hour. This washing does not remove the "combined" dye, but does take out that which is "uncombined." The smears were then placed in four changes of tertiary butyl alcohol. A small amount of dye comes out in the first change, hence it is inadvisable to leave slides in it for more than a minute or two. No dye comes out of the tissues in the next three changes. The smears may be left in them for from 15 minutes to an hour or longer, depending on their thickness. Allowance must be made for the fact that tertiary butyl alcohol penetrates about half as fast as ethyl alcohol. The slides were mounted directly in balsam, or passed thru two changes of xylene before mounting in balsam.

[1]Cole, E. C. 1936. A new methylene blue technic for permanent preparations. Stain Techn., **11**, 45.

The above technic should be more successful in the dehydration of tissues stained *intra vitam* with methylene blue than the procedures heretofore used, since it extracts considerably less dye.

Smears of *Trichonympha* stained in aqueous solution with a number of other dyes were also dehydrated in tertiary butyl alcohol. The loss of dye was very slight or nil with toluidine blue 0, Nile blue sulfate, eosin Y, Ponceau 2R and orange G. Fair results were obtained with crystal violet.

The use of tertiary butyl alcohol as a general dehydrating agent was developed by Johansen[2] in a series of articles. It has all the advantages of dioxane, and is moreover both cheap and non-toxic. It is miscible with water, alcohol, balsam and paraffin. It should be borne in mind, however, that the freezing point of tertiary butyl alcohol is about 25° C., and this dehydrating agent must, therefore, be kept in a fairly warm place.

[2]Johansen, D. A. 1937–1938. Tertiary butyl alcohol methods. El Palo Alto News, **1**, 1–3, 5–6, 13–14, 21–22; **2**, 1–4.

A MODIFIED STAIN AND PROCEDURE FOR TREMATODES

W. Carl Gower, *Game Division, Michigan Dept. of Conservation, Lansing, Michigan*

Trematodes are fairly easy to prepare as whole mounts, and there are a number of good staining methods by which they may be stained. The results usually desired are complete staining of the organs without stain in the parenchyma and muscle. If properly stained a sharp picture of the morphology and relationships of the organs results. It is unfortunate that stains and staining procedures are inconsistent, giving at one time and with one species of worm an excellent, sharp stain, and at another time or with another species a muddy, diffuse stain. The staining procedure described here has been found, over a period of two years, to give excellent and consistent results. It is sharper and less diffuse than any of the other carmine stains I have used, and is much more consistent than the hematoxylins. The stain is made up as follows:

To 100 cc. of 45% glacial acetic acid add 10 g. of carmine. Dissolve by heating and allow to come to a boil. Cool and filter. Carefully remove the filter paper from the funnel and spread it out flat to dry. The filtrate is Schneider's aceto-carmine and may be saved for the uses indicated for this stain. When the residue left on the filter paper is quite dry, remove it as carefully as possible. It may be used at once or placed in a tightly closed container for future use.

The staining solution is made up the same as Mayer's carmalum, substituting the acidified carmine described above for carminic acid.

Acidified carmine	1 g.
Alum	10 g.
Distilled water	200 cc.

The ingredients are mixed and dissolved by the aid of heat. When they are completely dissolved and cooled, filter the mixture and add a crystal of thymol to prevent mold growth.

If correctly used this stain gives a deep rose red color to the principal organs of trematodes. It is purely a nuclear stain, and consequently there is no diffuse stain in the cytoplasm, making the organs stand out in the practically transparent body. For best results the following procedure is suggested:

1. After the worms have been fixed and washed, if not already in water, they should be brought to water or not over 20% alcohol.
2. Place in the modified stain for 12–36 hours, depending on the size of the worm.
3. Remove from the stain and wash in one or two changes of water.
4. Dehydrate thru 20%, 35%, 50% to 70% alcohol.
5. The destaining is done in 70% alcohol, and is a very important step.

The best method I have found is the chlorine method of Mayer. This is done by placing a few crystals of potassium chlorate in the bottom of a small covered dish to which a few drops of concentrated hydrochloric acid are added. When chlorine is being given off from the mixture, fill the dish with 70% alcohol. This chlorinated alcohol is then used to destain the specimens. If deeply over-stained, the specimen may be immersed in the chlorinated alcohol, but for small specimens it is usually better to add the chlorinated alcohol to the alcohol covering them. In this way almost complete control is maintained over the destaining at all times. When adding the chlorinated alcohol to the specimen dish, care must be taken to disperse it evenly thruout the dish.

6. When the specimen is sufficiently destained (which can be determined only by trial and experience) remove the alcohol containing the chlorine and replace it with fresh 80% alcohol.

7. Finish the dehydration in the usual manner, and when completely dehydrated, add cedar wood oil to the absolute alcohol until the mixture is at least one half cedar oil.

8. Clear in cedar wood oil and mount in balsam.

LABORATORY HINTS

FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

The abstracts given here are intended primarily for laboratory use; consequently the technic in each instance is given in as much detail as possible.

J. A. de Tomasi — *Abstract Editor*

MICROSCOPE AND OTHER APPARATUS

RICHARDS, O. W. **A nomogram for the resolving power of microscope objectives.** *Trans. Amer. Micr. Soc.*, **57**, 316–18. 1938.

The nomogram consists of three scales: the wave length of light from 130–800 λ and the strong lines of the metals from cadmium to hydrogen, the numerical apertures from 0.07–2.0, and the resolving power of the system expressed in lines per inch and in microns. The scales are so placed that a straight line from the light employed thru the numerical aperture of the objective shows the resolving power. The value of monochromatic light in resolution becomes at once apparent. This is a convenient nomogram for anyone interested in critical microscopy.—*Virgene Warbritton Kavanagh.*

PHOTOMICROGRAPHY

BISHOP, F. W. **Exposure meter for cinephotomicrography and still photomicrography.** *Science*, **87**, 238–9. 1938.

Useful information is given on a scheme of setting up a photoelectric cell-galvanometer system. It is designed to gauge the light available in a microscope and to indicate the proper exposure. The cell is mounted in a fitting which slides over the microscope tube and holds it flat against it, or the ocular lens. All light is thus intercepted. Total cost of such unit is less than $30.—*J. A. deTomasi.*

MICROTECHNIC IN GENERAL

COLE, E. C. **Methyl methacrylate as a laboratory tool.** *Science*, **87**, 396–8. 1938.

This artificial plastic is insoluble in water, nearly insoluble in ethyl alcohol, amyl acetate, xylene, but readily soluble in chloroform and dioxan. A dioxan solution has been prepared which is colorless and can be used as a mounting medium. It dries more quickly than the common resins, and does not show discoloration on aging. No harmful action on stains has been observed and owing to its refractive index of 1.50–1.52, visibility is entirely satisfactory.

Rods of this material have found application as substitutes for expensive quartz rods in the illumination of living tissues. A rod ½ in. thick, tapered at the distal end and inserted in a lamp housing of a small 50 c.p. automobile bulb, furnishes enough illumination to permit examination of blood circulation in the vascular system of various organs.—*J. A. de Tomasi.*

CORDTS, H. M. **"Props" for cover glasses.** *Science*, **88**, 194. 1938.

In the preparation of embryo, or small insect slides, propping up the cover slip can be accomplished by using small bone curtain rings. They come in standard sizes fitting the usual round cover glasses. The ring is ground flat on both sides, and treated as a specimen ready for mounting. Wash in dist. water, run thru the alcohols, xylene, and soak and store in thin balsam. Before making a mount, let the ring adhere to the glass slip for 24–48 hr. After partial drying of the finished mount, paint the ring with heavy balsam.—*J. A. de Tomasi.*

MONK, C. R. **An aqueous medium for mounting small objects.** *Science*, 88, 174. 1938.

In this paper a medium is suggested which is capable of allowing mounting of minute parts of marine copepods so that an oil immersion lens can be used on them. It is a modification of Zirkle's Karo medium, as follows: white Karo syrup, 5 cc.; Certo, 5 cc.; water, 3 cc.; 1 crystal of thymol. When mounting, this medium begins to set in about 2 min.; regulate the velocity of setting by varying the water content. Dry by heat, and secure the cover glass with a drop of euparal.—*J. A. de Tomasi.*

DYES AND THEIR BIOLOGICAL USES

HAURY, V. G. **Modification of the titan yellow method for the determination of small amounts of magnesium in biological fluids.** *J. Lab. & Clin. Med.*, 23, 1079–84. 1938.

The author proposes a technic which eliminates objections to the older method based on Kolthoff's observation that Mg imparts a red color to alkaline solutions of titan yellow. In this proposed method, the determination of Mg depends upon a comparison between the excess titan yellow remaining in a standard and an unknown solution. He outlines methods for determining Mg in water, blood serum, and urine. The procedures are too detailed to be abstracted. In general, proteins are first removed by precipitation with trichloracetic acid; then Ca is removed with sodium oxalate. The dye is then added, mixed, centrifuged; the supernatant fluid is collected and the color compared with that of a similarly treated standard.—*P. R. Beamer.*

ANIMAL MICROTECHNIC

BANK, O. and KLEINZELLER, A. **Die Vitalfärbung des Fibroblastenzellkernes mit Kristallviolett.** *Arch. exp. Zellforsch.*, 21, 394–9. 1938.

The basic observation underlying this method of vital staining with crystal violet is the fact that the nucleus must be optically differentiated before it visibly begins to take up the dye. It is also possible that, from analogy of the theory of coacervation systems, optical differentiation or non-differentiation may be the result of a dehydration-hydration mechanism, whereby non-visibility correlates with maximum hydration of the nuclear colloids. The staining method is carried out in cover glass cultures of fibroblasts from the perichondrium of chicken embryo. It is found that crystal violet dissolved in the culture plasma is not adapted for vital staining even at 1:2,000,000 dilution. But the addition of electrolytes will permit staining, best results being obtained with Ca^{++}, and $SO_4^{=}$. This vital stain is reversible: it is obliterated by NH_4CNS, restored by $CaCl_2$. The nuclear stain is most successful when the nucleus is in telophase, the cells contain fat granules, and the cultures are two days old.—*J. A. de Tomasi.*

BRAGG, A. N. **The organization of the early embryo of Bufo cognatus as revealed especially by the mitotic index.** *Zts. Zellforsch. Mikr. Anat.*, 28, 154–78. 1938.

Yolky embryos section rather easily when embedded by the following modification of Hamlett's anilin method: Transfer embryos from 70% alcohol to aniline C.P., 1 part, 70% alcohol, 2 parts (2–6 hr.); anilin, 2 parts, 95% alcohol, 1 part, (2–6 hr.); anilin until clear, (approx. 1 hr.); 50% anilin, 50% toluene (1–6 hr.); 100% toluene, (1–3 hr.); sat. soln. 53° paraffin in toluene (1–4 hr.); melted 53° paraffin, 3–4 hr.; embed in 53° paraffin containing 1% bayberry wax if desired.—*Virgene Warbritton Kavanagh.*

BUCHER, O. **Untersuchungen über den Einfluss verschiedener Fixationsmittel auf das Verhalten des Schielddrüsenkolloids.** *Zts. Zellforsch.*, 28, 359–81. 1938.

The behaviour of the thyroid gland colloids of several mammalians, especially the rabbit, is investigated with particular reference to fixation in abs. alcohol, formalin, sublimate, picric acid, and OsO_4. It is found that fixation does not only affect the degree of colloidal shrinkage (and therefore capacity of staining) but reveals for each fixative definite shrinkage patterns. Fixation also influences

the appearance of vacuolization in the gland tissue: on this basis a differentiation is suggested between primary and secondary vacuoles. Staining of the marginal vacuoles has not succeeded. The isoelectric point of the thyroid colloids is also influenced by fixation: with formalin, pH 3.5; with alcohol, pH 4; with sublimate, pH 4.5; with picric acid, pH 5.5. A distinction between basophilic and acidophilic colloids cannot be established.—*J. A. de Tomasi.*

CHAUVIN, R. **La réaction de Feulgen-Verne chez le Criquet pélerin.** *Compt. Rend. Soc. Biol.*, **127**, 1075–6. 1938.

An account is given of the use of the Feulgen-Verne stain in demonstrating the organs of insects. The technic followed is not described. All nerve elements except the ganglia are colored deep violet. This may be evidence that the nerves of insects are myelinated. The Malpighian tubules are readily stained, certain of the tubes staining more deeply than others. In the alimentary canal, the musculature of the crop and gizzard and of the rectum between the rectal glands, the epithelia of the stomach, gastric caeca, and colon are stained. The illeum remains unstained. The deep staining reaction of the epithelia seems to uphold the common opinion that the digestion and absorption of fat begins in the stomach and gastric caeca. The pericardial cells are weakly stained, the stain being diffused in the protoplasm. The thoracic muscles stain strongly and the abdominal weakly. The fat body and hypodermis do not stain.—*F. J. Trembley.*

COCKE, E. C. **A method for fixing and staining earthworms.** *Science*, **87**, 443–4. 1938.

Histological slides of earthworms are often not quite as satisfactory as they should be. The following method of preparation is given: Clean the live specimen in water, feed (under moist cover) corn meal and agar 1:1; finely chopped lettuce may also be added. Change dish and food at intervals for 3 days. Cut specimen in ¾ in. portions and drop into 50° C. Allen's B-15. Fix 12 hr., rinse, and run thru 35, 50, 70, 80, 95, and 2 changes of 100% alcohol. Leave 1 hr. in each glass. Pass thru the following chloroform-alcohol combinations: 1:3, 1:1, 3:1, and chloroform, 1 hr. in each. Saturate chloroform with paraffin, leave 12 hr., add more paraffin, and put in oven at 58° C. for 6 hr. Pour off the liquid, add melted paraffin, and leave in oven 48 hr. Embed, and section at 12μ.

Stain 10–30 min. in Delafield's hematoxylin, wash, destain in 50% acid alcohol to a deep pink color, wash in NH_3-alcohol (5 drops NH_4OH to 100 cc. 50% alcohol) to a light blue color. Run up and mount as usual.—*J. A. de Tomasi.*

JOHNSTON, M. **Some methods of preparing teleost fish otoliths for examination.** *J. Royal Micro. Soc.*, **58**, 112–9. 1938.

Most otoliths require some degree of grinding on the convex surface before zones can be detected in them. For surface grinding and polishing, they are mounted, preferably with balsam, on the rounded end of a glass rod. The actual grinding is done with a razor hone made from Solenhofen lithographic slate; the polishing, with jeweler's rouge. Examination of otoliths, particularly thru the surfaces, is aided by the use of proper media of high refractive index. Best results are obtained with anilin (r.i. 1.580), aniseed oil (r.i. 1.557), and aq. solutions of chloral hydrate.

Sections of otoliths are prepared by a slow process of grinding, comparable to methods used with petrified paleontological specimens. A special grinder is here described suited for this type of material.—*J. A. de Tomasi.*

LISA, J. R. **A combined Gram-methyl-green-pyronin stain for formalin fixed tissue.** *Arch. Path.*, **26**, 728. 1938.

A method of staining as reported by Scudder and Lisa (Stain Techn., **6**, 1931) is brought up to date. The buffered crystal violet solution and National Aniline methyl green and pyronin dyes have been found stable and reliable for routine staining of paraffin sections. The work was performed with a batch of methyl green certified under the number NG-10 in 1932. Formulae and results are given in detail.—*Sara A. Scudder.*

LISON, L. **Etudes histophysiologiques sur les tubes de Malpighi des insectes. III. L'élimination des colorants basiques chez les Orthopteres.** *Zts. Zellforsch. Mikr. Anat.*, **28**, 179–209. 1938.

This is one of a series of papers dealing with the reactions of living cells to various dyes. Dyes in concentrations from 0.05–0.5% were injected into the hemocoeles of three species by means of a fine glass pipet inserted between two abdominal segments. Basic dyes were more toxic than acid dyes, and smaller doses were used to avoid pathological changes. The animals were killed 2 min. to 2 hr. after injection. The tubes were examined immediately, in place, with a binocular and then studied immersed in mammalian Ringer soln. or hemocoele fluid on a covered slide.

Methyl green and malachite green were decolorized so rapidly in the animal that their distributions could not be followed. Thionin (Gr), brilliant cresyl blue (Gr), methylene blue (RAL), methylene green, toluidine blue (Gr), neutral red (microcolor), and Nile blue (Gr) stained only the vital granules in the tubes. Basic fuchsin (Gr), neuviolette 3R (C), crystal violet (Gr), and safranin O (Gr) stained the cytoplasm diffusely. Chrysoidine (Gr) and Janus green B (Meist) stained both granules and cytoplasm. The species varied in the staining gradients along the tubes, but showed similar gradients of reduction and metachromacy.—*Virgene Warbritton Kavanagh.*

VAN ROOYEN, C. E. **The micromanipulation and microdissection of the molluscum contagiosum inclusion body.** *J. Path. & Bact.*, **46**, 425–36. 1938.

To make the tissue smear adhere to a cover slip, rub a few flakes of trilaurine onto the glass with an artist's paper pencil; pass the cover slip rapidly thru a Bunsen flame 3 times, wiping off excess grease with silk; then smear the fresh, moist tissue across the glass.—*S. H. Hutner.*

PLANT MICROTECHNIC

BHADURI, P. N. **Root-tip smear technique and the differential staining of the nucleolus.** *J. Royal Micro. Soc.*, **58**, 120–4. 1938.

The paper discusses the examination of smears of cellular contents for cytological or other purposes. Heitz's Nukleal-Quetschmethode relies advantageously upon the use of the Feulgen reaction as a selective stain; but the chrom-osmic fixation preceding it is criticized. A new way of studying root tip smears is found in the use of a differential nucleolus staining technic. During development of this method various observations were made: for each plant, the optimum concentration of acetic acid and formalin as fixatives should be determined experimentally; with most plants, acetic alcohol (1:3) is quite a satisfactory fixative for the Feulgen stain, but interferes with the nucleolar stain; duration of hydrolysis is a critical point to be determined beforehand.

Smear Technic: Method I. Fix overnight in Navashin's, or acetic alcohol (1:3). Wash, and transfer to stoppered tubes containing *N* HCl. Transfer tubes for definite lengths of time to water at 58–60° C. Wash with dist. water, and hold in de Tomasi's decolorized fuchsin (Stain Techn., **11**, 137–44, 1936) 30 min. to overnight. Smear each root tip in a drop of 45% acetic acid between slide and cover glass. After 5 min. loosen cover glass by immersing horizontally in acetic alcohol 1:1. Transfer for 5 min. to acetic alcohol 1:9; soak 10 min. in two changes of 95% alcohol; treat 1 hr. in sat. soln. of Na_2CO_3 in 80% alcohol. Rinse, and keep 10 min. in 70% alcohol. Stain 15 min. with light green (Semmens and Bhaduri, Stain Techn., **14**, 1, 1939). Differentiate under the microscope in following solution: sat. Na_2CO_3 in 80% alcohol, 10 cc.; 80% alcohol, 40 cc. Rinse in 70%, dehydrate in 95, 100% alcohol. Clear in xylene alcohol, xylene, and mount in neutral balsam.

Method II. Hydrolize material stored in 80% alcohol with: 95% alcohol and conc. HCl, 1:1. Bring gradually to water, and stain as above.—*J. A. de Tomasi.*

CROSS, G. L. **An improved method of staining with fast green.** *Proceedings Oklahoma Acad. Sc.*, **17**, 69–70. 1937.

This procedure, developed for anatomical study of plant material is claimed to avoid plasmolysis, under- or overstaining, and loss of time. Kill and fix 24 hr. in following solution: formalin, 5%; glacial acetic acid, 5%; 70% alcohol, 90%. Wash 2 hr., with one change, in 80% alcohol. Run thru the following alcohol series: 1 hr. in 80% ethyl, 65%, and tertiary butyl, 35%; 1 hr. in 90% ethyl, 45%, and butyl, 55%; 1 hr. in abs. ethyl, 25%, and butyl, 75%; 2–3 hr. in butyl, changing each hour. Infiltrate overnight on top of oven. Replace butyl alcohol with 6 changes of paraffin in 6 hr. Pass slides thru: xylene, 6 min.; abs. alcohol, 2 min.; 95% alcohol, rinse 5–6 times; 50% alcohol, rinse 5–6 times. Stain 6–12 hr. in 1% safranin in 50% alcohol. Wash in water and run thru: 50% alcohol, 2–3 min.; 70, 95, 100% alcohol, 40% abs. alcohol and 60% xylene, 5–6 rinses in each. Counterstain 2–4 min. with a few cubic centimeters of sat. clove oil soln. of fast green in 40% abs. alcohol and 60% xylene. Rinse 5–6 times in 40% abs. alcohol and 60% xylene, clear 5 min. in xylene, and mount.—*J. A. de Tomasi.*

GRIGG, F. C. **On the use of dioxan as a dehydrating medium.** *J. Queckett Micro. Club.* Series 4, **1**, 4–11. 1938.

For cytological material from plants, the following dioxan schedule has proved of advantage: 10%, 1 hr.; 20%, 2 hr.; 30%, 4 hr.; 40%, overnight; 50%, 4–5 hr.; 60%, 4–5 hr.; 70%, 24 hr.; 80%, 24 hr.; 90%, 24–36 hr.; 100%, 48 hr. After the last bath, introduce $CaCl_2$ for dehydration. Remove to another vessel with fresh dioxan at 25° C. and add a few drops of xylene every 15 min. occasionally removing part of the mixture. In 6–7 hr. material will be in pure xylene. Infiltrate with low melting point paraffin: beeswax, 1 part; paraffin, 6–10 parts at from 46° to 48° C. Cutting must be done at about 52° F.—*J. A. de Tomasi.*

PALMQUIST, E. M. **The simultaneous movements of carbohydrates and fluorescein in opposite directions in the phloem.** *Amer. J. Bot.*, **25**, 97–105. 1938.

Uranin is used in order to determine whether two different solutes can move simultaneously in opposite directions in the same phloem tissue. This water soluble Na salt of fluorescein is chosen instead of other fluorescein compounds because it seems to exercise no harmful effect on phloem tissue. The dye is introduced by immersing the terminal leaflet half way in a 0.1% aq. soln. Control of the progress of the dye is accomplished by cutting cross sections of the petiole of the treated leaf, and examining microscopically for fluorescense in a beam of ultraviolet light. It is proved that carbohydrates (sugars) move out of leaves thru the phloem, while fluorescein is entering.—*J. A. de Tomasi.*

MICROÖRGANISMS

ASHBY, G. K. **Simplified Schaeffer spore stain.** *Science*, **87**, 443. 1938.

This is a technic intended to facilitate the staining of bacterial spores by beginners. Dry the smear over a Bunsen burner. Heat on steam bath until water condenses in droplets on bottom of slide. Flood with 5% aq. malachite green, and heat 1 min. Drop and rinse in cool water. Counterstain 30 sec. with 0.5% aq. safranin, and rinse again. The spores stain green; the vegetative forms, red.—*J. A. de Tomasi.*

BAILEY, H. D. **A practical stain for the spirochetes of syphilis and Vincent's angina.** *J. Lab. & Clin. Med.*, **23**, 960. 1938.

In cases where dark-field tests failed to reveal them, smears stained by this method show the organisms plainly. *Procedure:* A direct smear of syphilis serum or tissue juice is made on a clean slide. In case of Vincent's angina, the ulcerated material is spread in a loopful of water. Air dry and fix with heat. Cover with *N*/20 HCl, 10 sec.; wash in running water, 5 sec.; cover with Gram's iodine solution, 5–10 sec.; wash; apply anilin-gentian violet, 5–10 sec.; wash; apply Gram's iodine solution, 5–10 sec.; wash; apply anilin-gentian violet, 5–10 sec. Wash, blot and examine. Gentian violet without anilin oil may be used. By applying the iodine and gentian violet a third time a very deep color is secured. The stain is not permanent.—*P. R. Beamer.*

GOLDBERG, L. H. **A rapid method for staining blood smears. A modification of the original Wright technique.** *J. Lab. & Clin. Med.*, **23**, 959. 1938.

This modification of Wright's technic is employed where time is a factor. A thin to medium smear is made and placed across the edges of an ordinary drinking glass filled ¾ full of dist. water. Cover the smear with Wright's stain for about 10 sec. Ignite the stain and allow to flame 10–20 sec. By use of a small rod the flaming slide is then dropped into the dist. water. Remove, dry and examine. Nucleated cells stain deeply and in much more detail than in the original procedure. Red blood cells are stained poorly.—*P. R. Beamer.*

HENDEY, N. I. **An efficient technique for cleaning diatoms.** *J. Royal Micro. Soc.*, **58**, 49–52. 1938.

The classification of diatoms is based on the interpretation of the silica frustules that make up the skeleton of the organisms. Cleaning must remove both the coleoderm and the protoplasmic contents of the cells. The chemical cleaning is carried out in a closed apparatus intended to take care of both cleaning and bleaching, with the added advantage of neutralizing the obnoxious fumes evolved. The operation is based on the heating of diatom material with HNO_3 and H_2SO_4 in a closed flask, a very short time for fresh material, boiling 3–10 min. for fossil material. Upon cooling, a $KClO_3$ soln. is introduced with great caution. Products of interactions include Cl_2 which is subsequently neutralized by allowing it to mix with a Na_2SO_3-Na_2CO_3 soln.—*J. A. de Tomasi.*

MIHAELOFF, S. **Comparative research in the different methods of staining the gonococcus.** *Revue Medicale Francaise*, **18**, 609–717. 1937.

A brief description of the appearance, morphology and cultural requirement of the gonococcus are given, along with a detailed review of the Gram technic from time of Gram and Roux. Formulae for various counterstains, single stains such as Loeffler's, and double stains such as Unna-Pappenheim are given. Double staining is defined as one which colors the cells in contrast to the bacteria. Emphasis is laid upon stained films as a necessary aid to diagnosis of gonorrhoea; for this purpose the smears should be prepared either by dilution of thick pus with water or by smearing thinly streaked films. It was concluded that the Gram staining technic is of chief importance for differential diagnosis and control of other methods for staining exudates. Recognition of gonococci in films was facilitated by procedures such as that of Galli-Valerio, but procedures such as the original Pappenheim and Pick-Jakobsohn gave inconstant results. No specific stain for the gonococcus was found.—*Sara A. Scudder.*

PIEKARSKI, G. **Studies on the cytology of paratyphus and coli bacteria.** *Arch. Mikrob.*, **8**, 428–39. 1937.

For demonstrating the "nucleoid" contents of the cell of these bacteria the fresh smear on a slide was fixed and stained in aceto-carmine, or was fixed in acetic acid, osmic acid, Carnoy's or sublimate alcohol and subjected to the Feulgen reaction as follows: After fixation, rinse in dist. water. Hydrolize about 10 min. in *N* HCl at 60° C. Rinse in dist. water. Stain for about 1 hr. in fuchsin sulfurous acid with an excess of SO_2. Wash in SO_2 in water (about 10–15 min., changed twice). Run up thru alcohol, xylene, and Canada balsam.—*Merritt N. Pope.*

SHIH, L. **Über den Heterothallismus des Staubbrandes, Spacelotheca cruenta (Kühn) Potter, der Mohrenhirse Andropogon sorghum Brot.** *Arch. Mikrob.*, **9**, 167–92. 1938.

In order to study the cytology of the germination stages, chlamydospores of *S. cruenta* are germinated in a hanging drop of dist. water, 2% glucose or other nutrient soln., and treated as follows: Nearly dry the drop in the air and fix for at least 24 hr. in a single drop of the strong chromo-aceto-osmic-acid of Flemming, wash thoroly, dry, and place for 12 hr. in ether to extract all fats. Cover the culture with a soln. of 2% celloidin in a mixture of ether, 70%; abs. alcohol, 30%. Let liquid evaporate, mordant in 4% iron alum and stain in 0.5% hematoxylin.—*Merritt N. Pope.*

TABET, F. **A modification of Wilson and Blair's bismuth medium suitable for both typhoid and paratyphoid bacilli.** *J. Path. & Bact.*, **46**, 181–93. 1938.

This experimental study of the Wilson-Blair medium for the detection of typhoid and paratyphoid B as black colonies in the presence of many *B. coli* demonstrated that (1) the bismuth is mainly responsible for the blackening (due to Bi_2S_3), and also helps inhibit *B. coli;* (2) the selectivity is mostly due to the brilliant green.

The modified medium is prepared with 3 sterile flasks A, B and C: In A place 6 g. bismuth ammonio-citrate (Scales); in B, 10 g. anhyd. Na_2SO_3; in C, 10 g. glucose. To flask A add 50 cc. boiling dist. water and shake till the bismuth ammonio-citrate is dissolved; add 1 cc. 5*N* NaOH, mix well and cool. To B add 100 cc. boiling dist. water and cool. To C add 50 cc. cold dist. water; boil 1 min. and cool. Mix contents of A and B, add 3.5 g. anhyd. Na_2HPO_4, boil about 30 sec., cool, add contents to C. This stock mixture keeps for several months in the dark at room temperature. Shake thoroly before use.

The medium: To 100 cc. of melted nutrient agar at about 80° C. add 20 cc. of the stock mixture; 1 cc. of 8% $FeSO_4 \cdot 7H_2O$ in dist. water and 1 cc. of 0.5% brilliant green in dist. water, with vigorous shaking after each addition. Pour into plates and use as fresh as possible. The medium may be stored about 5 days in the refrigerator but deteriorates rapidly at room temperature. The pH is usually between 7.9–8.0; adjustment is unnecessary.—*S. H. Hutner.*

WINOGRADSKY, S. **Etudes sur la microbiologie du sol et des eaux (neuvième mémoire). Sur la morphologie et l'oecologie des Azotobacter.** *Ann. Inst. Pasteur*, **60**, 351–400. 1938.

A technic is offered to avoid overstaining the background when staining colonies of Azotobacter in soil on silica gel plates. Stain in 1% fast acid blue (violamine) (recommended by Romell, Stain Techn., **9**, 141. 1934), phenol 0.5% for about 30 sec. This acts more as a mordant than a stain. Wash off first stain then apply a weak solution of "gentian" for 30 sec.

If the culture is old, containing cysts, heat the drop of acid blue until it just begins to steam, then stain 1 min. in "vesuvine", rinse, stain quickly with "gentian". In successful preparations the central body of the cyst is brown or black, the outer layer of the capsule is blackish, the inner yellow.

Mounting in balsam causes bad shrinkage. Instead, dry the slides, wrap in silk, and examine in water as required. Preparations keep their freshness.—*S. H. Hutner.*

STAINS RECENTLY CERTIFIED

In the table below is given a list of the batches of stains approved since the last one listed in the Oct. number of this journal.

STAINS CERTIFIED SEPT. 1, 1938 TO NOV. 30, 1938*

Name of dye	Certification No. of batch	Dye Content	Objects of tests made by Commission†	Date approved
Crystal violet	NC—19	90%	As histological, cytological, and bacteriological stain; and in bacteriological media	Sept. 26, 1938
Basic fuchsin	NF—31	88%	For general staining, the Feulgen reaction, and in bacteriological media	Sept. 30, 1938
Methylene blue	MA—1	85%	As histological and bacteriological stain; and as constituent of blood stains	Oct. 4, 1938
Nile blue A	LNb—1	73%	As stain for fats and fatty acids	Oct. 10, 1938
Phloxine	NPh—8	84%	For histological counterstain	Oct. 17, 1938
Methyl green	CG—8	60%	In histology and as constituent of Pappenheim stain	Oct. 19, 1938
Sudan IV	LZ—1	68%	As fat stain	Oct. 19, 1938
Crystal violet	BC—1	89%	As histological, cytological and bacteriological stain; and in bacteriological media	Oct. 20, 1938
Azure A	NAz—6	90%	As histological stain and as constituent of blood stains	Nov. 7, 1938
Fast green FCF	NGf—4	90%	As histological counterstain	Nov. 12, 1938
Acid fuchsin	NR—11	62%	As histological stain, and for use in Andrade indicator	Nov. 17, 1938
Indigo carmine	CI—5	90%	As histological stain	Nov. 30, 1938

*The name of the company submitting any one of these dyes will be furnished on request.

†It is not to be inferred that these are the only uses for which each of these samples may be employed. The Commission ordinarily tests each due for such of its common uses as seem to give the most severe check as to its staining value. Certification does not in any instance, however, imply approval for medicinal use.